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Erk Kinases Link Pre-B Cell Receptor Signaling to Transcriptional Events Required for Early B Cell Expansion

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SUMMARY

The pre-B cell receptor (pre-BCR) plays a crucial role in the development of immature B cells. Although certain aspects of proximal pre-BCR signaling have been studied, the intermediate signal transducers and the distal transcription modulators are poorly characterized. Here, we demonstrate that deletion of both Erk1 and Erk2 kinases was associated with defective pre-BCR-mediated cell expansion as well as a block in the transition of pro-B to pre-B cells. Phosphorylation of transcription factors Elk1 and CREB was mediated by Erk, and a dominant-negative mutation in the Erk-mediated phosphorylation sites of Elk1 or CREB suppressed pre-BCR-mediated cell expansion as well as expression of genes including *Myc*, which is involved in the cell-cycle progression. Together, our results identify a crucial role for Erk kinases in regulating B cell development by initiating transcriptional regulatory network and thereby pre-BCR-mediated cell expansion.

INTRODUCTION

B cell maturation can be divided into a series of developmental stages that are characterized by the differential expression of a variety of marker proteins and changes in the rearrangement status of the immunoglobulin heavy (*Igh*) and light (*Igl*) chain genes (Hardy and Hayakawa, 2001). *Igh* gene rearrangement is initiated during pro-B cell stage, and the resulting product in association with λ5 and VpreB forms a surface-expressed pre-B cell receptor (pre-BCR). Correct assembly of the pre-BCR triggers a signaling cascade that induces proliferation, downregulation of the pre-BCR complex, and differentiation into small pre-B cells. Subsequently, productive gene rearrangement at the *Igl* loci takes place, leading to synthesis of IgM on the surface of immature B cells (Meffre et al., 2000). Other pathways have been shown to participate in checkpoints during B cell development. Interleukin (IL)-7, a cytokine produced by stromal cells, was identified as a proliferation factor for the B cell lineage. Indeed, disruption of the IL-7 signaling pathway prevents the appearance of the pre-BCR-expressing population in the bone marrow (Hendriks and Middendorp, 2004).

Mice that are deficient in genes encoding components of the proximal pre-BCR signaling cascade have provided valuable insights into the nature of the pre-BCR signal. Signaling from the pre-BCR depends critically on the receptor-associated transmembrane molecules Igα and Igβ, in particular on their immunoreceptor tyrosine-based activation motifs (ITAMs) in the cytoplasmic domains (Meffre et al., 2000; Hendriks and Middendorp, 2004). After tyrosine phosphorylation of the Igα and Igβ ITAMs, the protein tyrosine kinase (PTK) Syk is recruited and subsequently activated. The binding of Syk to the phosphorylated Igα and Igβ also places the active Syk in the right position to allow further phosphorylation of neighboring ITAM sequences. This results in further Syk recruitment and activation (Jumaa et al., 2005). Then, activated Syk phosphorylates phospholipase C (PLC)-γ2, leading to the generation of the second messengers diacylglycerol (DAG) and inositol-1,4,5-trisphosphate (Kurosaki, 2002). The essential role of such an ITAM-Syk signaling circuit in B cell development has been underscored by impaired pre-B cell development in mice carrying a mutated Igα ITAM with a truncation of Igβ (IgαΔC IgβΔC, IgαFF IgβΔC) or in mice deficient in Syk, alone or together with Zap70 (Reichlin et al., 2001; Kraus et al., 2001; Turner et al., 1995; Schweighoffer et al., 2003). Another model has also emerged that proposes that the Syk-independent, but Src family PTKs (SFKs)-dependent, NF-κB signaling pathway participates in pre-B cell development. In fact, in Blk Fyn Lyn triple Src-PTK-deficient mice, B cell development is arrested prior to the pre-B cell stage (Saijo et al., 2003).

Although much has been learned as to the functions of the Igα and Igβ ITAMs, Src-family PTKs, and Syk-family PTKs, less is known about the potential connection between such PTKs and nuclear events that govern pre-BCR-mediated cell expansion and differentiation. In this regard, serine and threonine kinases downstream of the Syk-family and Src-family PTKs could be an attractive candidate because some of serine and threonine kinases are known to shuttle between the cytoplasm and the nucleus and/or to regulate transcription factors, thereby controlling

gene expression. Because Erk activation is dependent upon Syk in transformed B cells (Jiang et al., 1998), we hypothesized that Erk might interface Syk with nuclear events, thereby contributing to the pre-BCR-mediated responses.

To test this hypothesis, we have generated mice deficient in both Erk1 and Erk2. By using these mice, we show an essential role of Erk1 and Erk2 in the pre-BCR-mediated cell expansion as well as in the transition of pro- to pre-B cells. Erk1 and Erk2 activation is dependent on expression of Syk and Zap70. Activated Erk1 and Erk2 contribute to the phosphorylation of transcription factors such as Elk1 and CREB and their activation, which in turn regulate genes involved in cell expansion. Together, our study identifies Erk1 and Erk2 as key participants in the pre-BCR check point and suggests the potential mechanism by which Erk1 and Erk2 exert their function.

RESULTS

Erk1 and Erk2 Are Indispensable for a Transition of Pro-B to Pre-B Cells

Targeted disruption of Erk2 results in embryonic lethality due to defective placental development (Hatano et al., 2003). Thus, in order to examine the effect of the double deficiency of Erk1 and Erk2 on B cell development and function, we crossed *Mapk3*$^{-/-}$ *Mapk1*$^{fl/fl}$ (encoding Erk1 and Erk2, respectively) mice with *Mx-cre* mice that bear the *cre* transgene under the control of type I interferon-inducible Mx promoter (referred to hereafter as inducible-Erk double-deficient mice). Two weeks after p(I)p(C) injections, these mice showed a reduction in the number of pre-B cells (B220^{low}IgM^{-}CD43^{-}) to 7% of wild-type controls and a corresponding decrease in immature B cells (B220^{low}IgM^{+}CD43^{-}) in the bone marrow (Figures 1A and 1B). In contrast, Erk1-deficient (*Mapk3*$^{-/-}$ *Mapk1*$^{fl/fl}$) or inducible Erk2-deficient (*Mx-cre*$^{+}$ *Mapk3*$^{+/-}$ *Mapk1*$^{fl/fl}$) mice did not show such a drastic reduction of the pre-B or the immature B cell compartment after p(I)p(C) injection (Figures 1A and 1B). Pro-B cells can be further subdivided on the basis of surface expression of HSA and BP-1 into subfractions A, B, C, and C′. The BP-1^{+}HSA^{high} cells (fraction C′) represent cycling cells that are in the transition from pro-B to pre-B and that are in the process of downregulating surface CD43. We found that the ratio of BP-1^{+}HSA^{high} (fraction C′) cells to BP-1^{+}HSA^{low} (fraction C) cells in p(I)p(C)-treated inducible-Erk double-deficient mice was much lower than in other genotypes (Figure 1A), indicating a defect in the transition from pro-B to pre-B cells in these mice.

Deletion of the *Mapk1* genomic locus was assessed by quantitative PCR with primers that detect the *Mapk1* flox (*Mapk1*^{fl}) and null (*Mapk1*$^{\Delta}$) alleles. After p(I)p(C) injection, bone-marrow pro-B cells from inducible-Erk2-deficient or -Erk double-deficient mice showed efficient deletion in the *Mapk1*^{fl} allele of almost 95% (Figure 1C). However, recirculating B cells (B220^{high}IgM^{+}) in the bone marrow hardly deleted *Mapk1* (Figure S1A available online). Two weeks after p(I)p(C) injections, in the spleen of inducible-Erk double-deficient mice, ~90% of the immature B cells (B220^{+}IgM^{high}IgD^{low}) were lost (Figure S1B), and the remaining splenic B cells were also derived from cells that had escaped *Mapk1* deletion (Figure S1A).

Because Erk1 and Erk2 are widely expressed kinases in other cell types such as bone-marrow stromal cells, we next determined whether the observed defect in B cell development was due to an intrinsic lack of Erk1 and Erk2. Therefore, we reconstituted irradiated an alymphoid host deficient in recombination-activating gene 1 (Rag1) with bone-marrow cells from wild-type, Erk1-deficient, inducible-Erk2-deficient, or inducible-Erk double-deficient mice. Two weeks after p(I)p(C) injection, in contrast to other genotypes, inducible-Erk double-deficient bone-marrow cells were unable to reconstitute B cell development (Figures 2A and 2B). Together, these results show that the impaired early B cell development in the absence of Erk1 and Erk2 is cell autonomous.

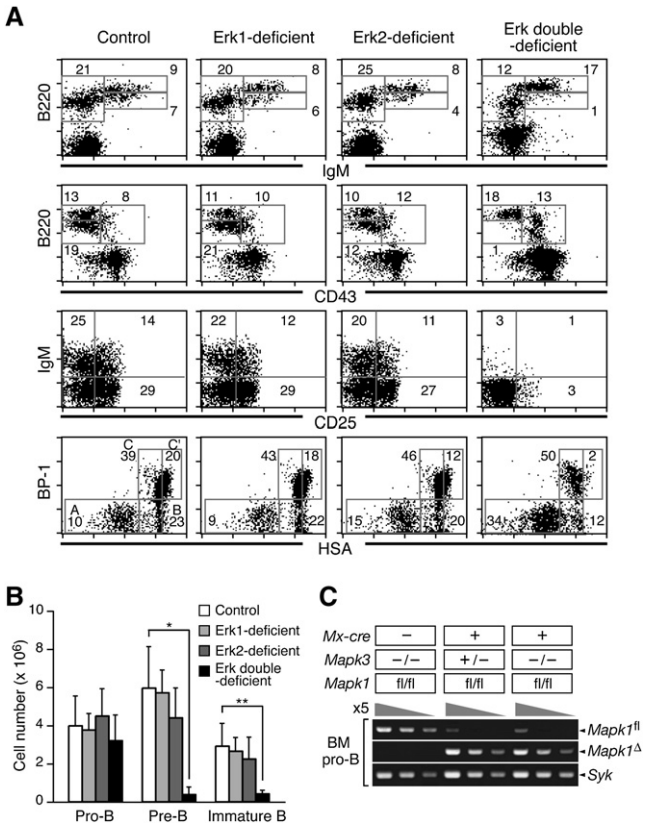

Figure 1. B Cell Development Is Blocked at the Pro-B Cell Stage in Inducible-Erk Double-Deficient Mice

(A) Representative FACS analysis of bone marrow from *Mx-cre*$^{+}$ *Mapk3*$^{+/+}$ *Mapk1*$^{+/+}$ (Control), *Mapk3*$^{-/-}$ *Mapk1*$^{fl/fl}$ (Erk1-deficient), *Mx-cre*$^{+}$ *Mapk3*$^{+/-}$ *Mapk1*$^{fl/fl}$ (inducible-Erk2-deficient), or *Mx-cre*$^{+}$ *Mapk3*$^{-/-}$ *Mapk1*$^{fl/fl}$ (inducible-Erk double-deficient) mice at day 14 after the first of three p(I)p(C) injections to all mice. Numbers represent percentages of cells within the live cell fraction (B220 versus IgM and B220 versus CD43), B220^{low} B cell fraction (IgM versus CD25) or B220^{+}CD43^{+} pro-B cell fraction (BP-1 versus HSA).

(B) Numbers of B cell subpopulations in bone marrow. Bars and error bars represent mean and SD of cells in two femurs and tibias per mouse. Pro-B, B220^{+}IgM^{-}CD43^{+}; Pre-B, B220^{low}IgM^{-}CD43^{-}; Immature B; and B220^{low}IgM^{+}CD43^{-} cells are shown. *p = 0.003, **p = 0.001.

(C) Genomic PCR analysis for determining the ratio of Mapk1-flox (*Mapk1*^{fl}) versus deleted (*Mapk1*$^{\Delta}$) alleles in bone-marrow pro-B cells (B220^{+}IgM^{-}CD43^{+}). Cells were prepared by FACS sorting from Erk1-deficient, inducible-Erk2-deficient, or inducible-Erk double-deficient mice at day 14 after the first of three p(I)p(C) injections. Genomic PCR for the *Syk* locus was performed as a loading control.

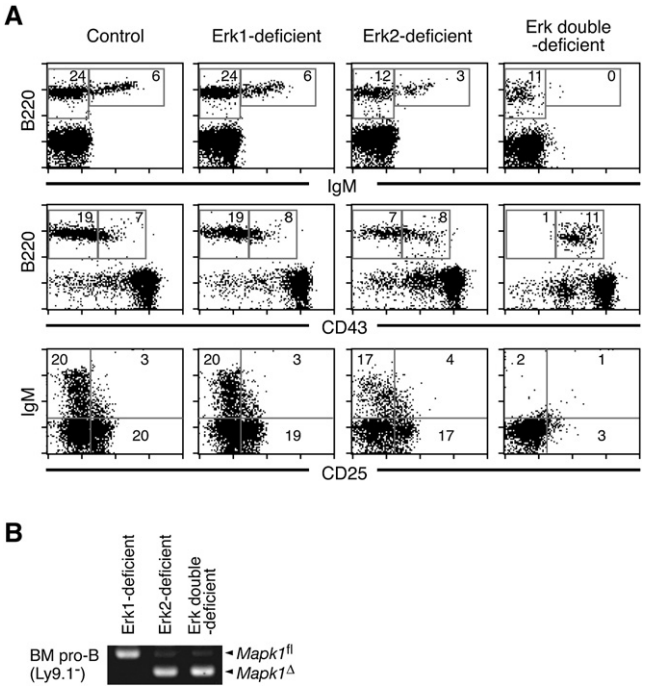

Figure 2. Impaired B Cell Development in Inducible-Erk Double-Deficient Mice Is B Cell Intrinsic

(A) Sublethally irradiated recipient mice (*Rag1*$^{-/-}$, Ly9.1^{+}) were reconstituted with bone marrow from *Mx-cre*$^{+}$ *Mapk3*$^{+/+}$ *Mapk1*$^{+/+}$ (Control), *Mapk3*$^{-/-}$ *Mapk1*$^{fl/fl}$ (Erk1-deficient), *Mx-cre*$^{+}$ *Mapk3*$^{+/-}$ *Mapk1*$^{fl/fl}$ (inducible-Erk2-deficient), or *Mx-cre*$^{+}$ *Mapk3*$^{-/-}$ *Mapk1*$^{fl/fl}$ (inducible-Erk double-deficient) mice. Recipient mice were analyzed at day 14 after the first of three p(I)p(C) injections. Representative FACS analyses of the B cell population of donor origin cells (Ly9.1^{-}) in the bone marrow are shown. Numbers indicate the percentages of gated populations as described in the Figure 1 legend. All recipient mice (n = 4) showed similar phenotypes.

(B) Genomic PCR analysis to confirm the deletion of *Mapk1* flox allele in donor origin pro-B cells (Ly9.1^{-}B220^{+}IgM^{-}CD43^{+}). Cells were prepared by FACS sorting from Erk1-deficient, inducible-Erk2-deficient, or inducible-Erk double-deficient reconstituted mice at day 14 after the first of three p(I)p(C) injections.

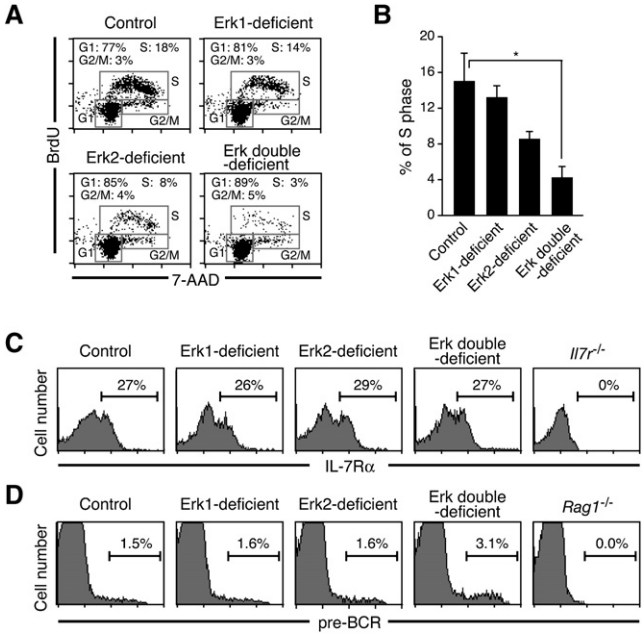

Figure 3. Mutation of Erk1 and Erk2 Does Not Perturb Surface Expression of IL-7 Receptor or Pre-BCR but Results in Impaired Cell Cycling

(A) Cell-cycle analysis of bone-marrow pro-B cells from p(I)p(C)-treated mice as in Figure 1. BrdU-pulsed bone-marrow cells were stained with antibodies to B220, CD43, and BrdU, then labeled with 7-AAD. Percentages of cells in G1, S, and G2/M phases are shown in the gate of B220^{+}CD43^{+} pro-B cells. Data are representative of four individual mice.

(B) Percentage of S phase cells in the gate of B220^{+}CD43^{+} pro-B cells as in (A). The data are given as mean ± SD from four individual mice. *p = 0.004.

(C and D) FACS analysis of bone-marrow cells from control, Erk1-deficient, inducible-Erk2-deficient, inducible-Erk double-deficient, *Il7r*$^{-/-}$, or *Rag1*$^{-/-}$ mice, showing histograms of expression of IL-7Rα (C) or pre-BCR (D) in B220^{+}CD43^{+} pro-B cells. Numbers show the percentage of cells within the region marked by a horizontal bar. The pre-BCR was detected with the SL156 antibody recognizing a conformational epitope on the surrogate light-chain-Igμ complex.

Requirement for Erk1 and Erk2 in Pre-BCR-Mediated Signaling

Because Erk kinases are known to be involved in the regulation of proliferation in other cell types such as fibroblasts, we examine the cell-cycle status of pro-B cells from various genotypes. As shown in Figures 3A and 3B, the proportion of pro-B cells that entered the S phase was considerably decreased in the p(I)p(C)-treated inducible-Erk double-deficient mice. Given the importance of expression of the pre-BCR and IL-7 receptor (IL7R) in cell-cycle progression of pro- and pre-B cells (Meffre et al., 2000; Hendriks and Middendorp, 2004), we reasoned that the above observation could be due to impaired expression of these receptors or defective signaling through the receptors. The former possibility is unlikely because cell-surface expression of both IL7Rα and pre-BCR was not impaired in p(I)p(C)-treated inducible-Erk double-deficient pro-B cells (Figures 3C and 3D). Before addressing the latter possibility, we tested whether Erk kinases are indeed activated after pre-BCR expression or IL-7 stimulation. Introduction of exogenous μH into *Rag1*$^{-/-}$ pro-B cells resulted in the formation of surface pre-BCR, and an increased phosphorylation level of the Erk kinases, demonstrating the connection between pre-BCR expression and Erk kinase activation (Figure 4A). Moreover, only marginal activation of the Erk kinases was observed in pro-B cells after IL-7 stimulation alone (data not shown).

To address the effects of Erk1 and Erk2 ablation on pre-BCR-mediated cell expansion, we compared the proliferation capacity of pro-B cells among various genotypes. As previously demonstrated (Fleming and Paige, 2001), compared with wild-type pro-B cells, inability of expression of pre-BCR complex on the surface of pro-B cells such as μMT or *Rag1*$^{-/-}$ resulted in a severe reduction in their proliferation at low concentration of IL-7 (Figure 4B). Such a defect in *Rag1*$^{-/-}$ pro-B cells could be restored by the enforced expression of μH to a level equal to that of wild-type cells, thereby establishing the requirement of pre-BCR for normal proliferation. Importantly, this proliferation was abrogated in pro-B cells deficient in both Erk1 and Erk2, but not in either Erk1 or Erk2 alone, and the proliferation was similar to that of μMT or *Rag1*$^{-/-}$ pro-B cells (Figure 4B). These results suggest that Erk kinases participate in pre-BCR-mediated cell expansion.

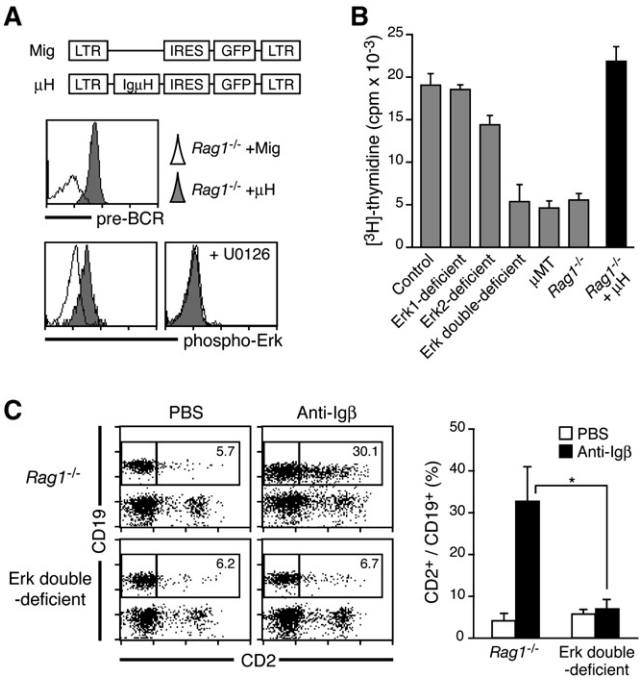

Figure 4. Defective Pre-BCR Signaling in Inducible-Erk Double-Deficient Pro-B Cells

(A) The rearranged μH gene was inserted into the retrovirus vector pMX-IRES-GFP. *Rag1*$^{-/-}$ pro-B cells were infected with the indicated retrovirus and cultured for 24 hr in the absence of IL-7 prior to FACS analysis of pre-BCR (SL156 antibody) or phosphorylated Erk. In the phosphorylated Erk analysis, MEK inhibitor U0126 (10 μM) was added to the culture for 12 hr. Results are shown in GFP^{+} gated cells.

(B) Proliferation was measured by [^{3}H]-thymidine incorporation after 2 days of culture of pro-B cells from the indicated mice. GFP^{+}-sorted *Rag1*$^{-/-}$ pro-B cells infected with μH were analyzed as the pre-BCR^{+} *Rag1*$^{-/-}$ control. A total of 0.16 ng/ml of IL-7 was added to the culture. The data show the mean of triplicate wells; error bars represent SD.

(C) FACS analysis of bone-marrow cells from *Rag1*$^{-/-}$ mice or p(I)p(C)-treated radiation chimeras reconstituted with inducible-Erk double-deficient bone-marrow cells, 9 days after injection with PBS or Igβ antibodies. In the upper panel, *Rag1*$^{-/-}$ mice (Ly9.1^{-}) were used as a control. In the lower panel, donor cells (Ly9.1^{-}) reconstituted with irradiated *Rag1*$^{-/-}$ mice (Ly9.1^{+}) were analyzed. CD19 versus CD2 plots are gated on Ly9.1^{-} cells so that host cells were excluded. Percentages of CD2^{+} among CD19^{+} cells in mice analyzed are shown (right panel). The data are given as mean ± SD from six individual mice. *p = 0.001.

In addition, Erk kinases play an important role in pro-B cell survival, as indicated by the observation that the number of dead cells was increased in p(I)p(C)-treated inducible-Erk double-deficient pro-B cells in the absence of IL-7 or low amount of IL-7 (Figure S2A). It has previously been demonstrated that the ability to proliferate in low IL-7 concentrations requires not only pre-BCR expression but also a signal downstream of the pre-BCR and that downstream of the pre-BCR, the Erk pathway is essential in order to mediate this effect (Fleming and Paige, 2001). Indeed, concordant with this notion, p(I)p(C)-treated inducible-Erk double-deficient pro-B cells exhibited a response to IL-7 similar to that of *Rag1*$^{-/-}$ or μMT pro-B cells (Figure S2B).

Defective In Vivo Signaling from Igβ in the Absence of Erk1 and Erk2

To examine directly whether Erk kinases transmit the pre-BCR signals and thereby are essential for the pro-B to pre-B cell developmental transition, we used a previously established model in which injection of Igβ antibody into *Rag1*$^{-/-}$ mice was able to induce differentiation of pro-B to pre-B cells (Nagata et al., 1997). As expected, injection of Igβ antibody into *Rag1*$^{-/-}$ mice led to a large increase in pre-B cell number; however, no such developmental restoration was seen in irradiated mice reconstituted from p(I)p(C)-treated inducible-Erk double-deficient bone-marrow cells (Figure 4C). This result reinforces the idea that Erk1 and Erk2 are required to transduce an Igβ signal, which presumably mimicks the pre-BCR signal, thereby participating in transition from pro-B to pre-B cells.

Constitutively Active MEK Rescues Expansion Defect of Syk Zap70 Double-Deficient Pro-B Cells

Consistent with a previous report (Schweighoffer et al., 2003), *mb1-cre*$^{+}$ *Syk*$^{fl/fl}$ *Zap70*$^{-/-}$ mice (Syk Zap70 double-deficient mice, hereafter) (Figure S3) showed a complete block at the fraction C stage in pro-B cells (Figure 5A). The similar developmental arrest in p(I)p(C)-treated inducible-Erk double-deficient bone-marrow B cells prompted us to test the hypothesis that the Erk kinases function downstream of Syk and Zap70, thereby participating in pre-BCR-mediated expansion. Supporting this hypothesis, Erk activation was dependent on expression of Syk and Zap70 because increased phosphorylation of Erk was seen in μMT control pro-B cells stimulated by anti-Igβ crosslinking, but not in Syk Zap70 double-deficient pro-B cells (Figure 5B). Cell-surface expression of Igβ between μMT and Syk Zap70 double-deficient cells was similar (data not shown).

Next, we tested whether a constitutive active form of MEK (MEK-CA), an Erk-activating kinase, can overcome defects due to the loss of Syk and Zap70. Transduction of a retrovirus encoding Syk into Syk Zap70 double-deficient pro-B cells induced a robust expansion in the presence of a low amount of IL-7. Under these culture conditions, μMT pro-B cells never expanded unless transduced with μH, indicating that the expansion observed is pre-BCR dependent. Importantly, expression of MEK-CA in Syk Zap70 double-deficient pro-B cells corrected the expansion defect due to the loss of Syk and Zap70 (Figure 5C). Hence, Erk activation probably occurs downstream of Syk and Zap70 and is crucial for pre-BCR-mediated cell expansion.

Pre-BCR-Induced Cell Expansion Is Controlled by Erk-Dependent Gene Expression

In fibroblasts and PC12 cells, many growth factors elicit expression of a class of genes known as immediate-early genes (IEGs), several of which encode transcription factors that regulate transcription of genes directly involved in cell-cycle progression such as the *Ccnd1* gene (Roovers and Assoian, 2000). Thus, we reasoned that Erk promoted pro-B cell expansion by regulating the mRNA expression of such IEGs or other transcription-factor-encoding genes. We therefore sought transcription factors that are induced after pre-BCR stimulation in an Erk-dependent manner and that might influence pro-B cell expansion. To obtain information of the transcription factors upregulated by the Erk activation, we performed microarray analysis with μMT pro-B cells. RNA was isolated from μMT pro-B cells unstimulated or stimulated by anti-Igβ for 0.75 hr or 6 hr in the presence or absence of MEK inhibitor and hybridized to arrays representing 39,000 markers. Of the genes assayed, 171 genes,

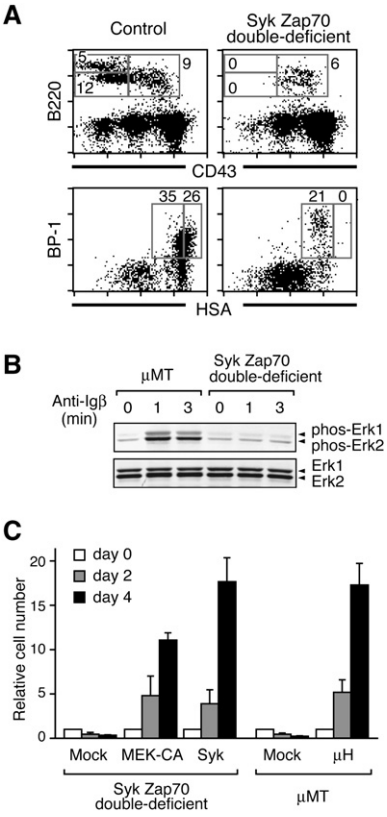

Figure 5. Constitutively Active MEK Can Bypass the Expansion Defect of Syk Zap70 Double-Deficient Pro-B Cells

(A) FACS analysis of bone marrow from *mb1-cre*$^{+}$ *Syk*$^{+/+}$ *Zap70*$^{+/+}$ (Control) and *mb1-cre*$^{+}$ *Syk*$^{fl/fl}$ *Zap70*$^{-/-}$ (Syk Zap70 double-deficient) mice. Numbers represent percentages of cells within the live cell fraction (B220 versus CD43) or B220^{+}CD43^{+} pro-B cell fraction (BP-1 versus HSA).

(B) Anti-Igβ-induced Erk1 and Erk2 phosphorylation. CD19^{+} pro-B cells from μMT or Syk Zap70 double-deficient mice were stimulated with Igβ antibody for the indicated times. Whole-cell lysate was separated by SDS-PAGE and immunoblotted with phospho Erk (phos-Erk1, phos-Erk2) or Erk (Erk1, Erk2) antibodies.

(C) CD19^{+} pro-B cells from Syk Zap70 double-deficient or μMT mice were infected with the indicated retrovirus. The data show the kinetics of relative cell number for each GFP^{+} transfectant during 4 days of culture in the presence of 0.01ng/ml of IL-7. The data are presented as the mean ± SD of triplicate.

including 22 transcription factors, were scored as differentially upregulated in an Erk-dependent manner either 0.75 hr or 6 hr after anti-Igβ stimulation (Figure S4A). The expression of these candidate transcription factors was further validated by quantitative RT-PCR analysis, and 16 genes were confirmed as reproducibly and substantially changed (Figures S4B and S4C). These genes include IEGs such as *Egr1*, *Egr2*, *Egr3*, *Myc*, and *Fos*, the expression of which showed a transient increase in an Erk-dependent manner after anti-Igβ stimulation.

To evaluate the role of these transcription factors in expanding pro-B cells, we took a rescue approach in which overexpression of these transcription factors can bypass the expansion defects linked by loss of Erk1 and Erk2. Because in gene-targeting studies *Xbp1* and *Bach2* were previously reported as dispensable genes for early B cell differentiation (Reimold et al., 2001; Muto et al., 2004), we focused on testing the remaining 14 genes.

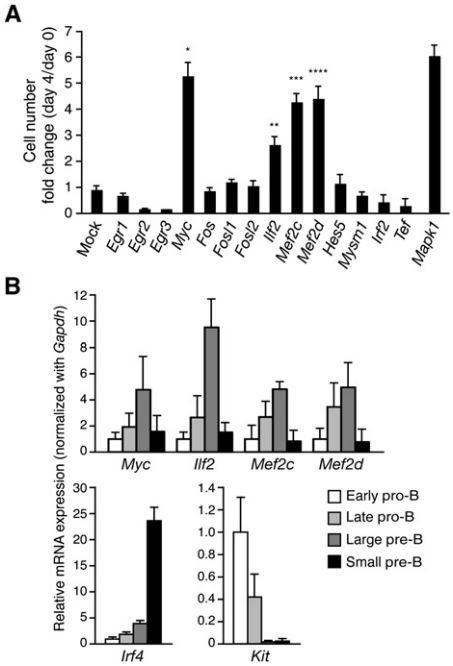

Figure 6. Identification of the Expansion-Promoting Genes Activated by Erk

(A) B220^{+}CD43^{+} pro-B cells from p(I)p(C) treated inducible-Erk double-deficient mice were infected with the indicated retrovirus. The data show the fold change of the cell number for each GFP^{+} transfectant during 4 days of culture in the presence of 0.01 ng/ml of IL-7. *p = 0.0002, **p = 0.0015, ***p = 0.0001, and ****p = 0.0004, compared with mock transfectant. Data are representative of at least three independent experiments and shown as the mean ± SD of triplicate.

(B) Bone-marrow subpopulations of early pro-B (B220^{+}CD43^{+}HSA^{low}IgM^{-}IgD^{-}), late pro-B (B220^{+}CD43^{+}HSA^{high}IgM^{-}IgD^{-}), large pre-B (B220^{+}CD43^{low}HSA^{high}IgM^{-}IgD^{-}FSC^{high}), and small pre-B (B220^{+}CD43^{low}HSA^{high}IgM^{-}IgD^{-}FSC^{low}) were prepared by FACS sorting. Total RNA was analyzed by quantitative PCR, with the expression of *Myc*, *Ilf2*, *Mef2c*, *Mef2d*, *Irf4*, or *Kit* normalized to that of *Gapdh*. Data are representative of at least three independent experiments and shown as the mean ± SD of triplicate.

Among them, only four transcription factors, *Myc*, *Ilf2*, *Mef2c*, and *Mef2d*, were able to restore the cell expansion of p(I)p(C)-treated inducible-Erk double-deficient pro-B cells (Figure 6A), suggesting that downstream of Erk, these four transcription factors may play a role in the cell expansion during the pro- to pre-B cell transition. In further support of this possibility, *Myc*, *Ilf2*, *Mef2c*, and *Mef2d* transcript expression was increased in large cycling pre-B cells but subsequently downregulated in small resting pre-B cells. Consistent with previous reports (Muljo and Schlissel, 2003; van Zelm et al., 2005), expression of *Kit* was downregulated in large cycling pre-B cells, whereas that of *Irf4* was upregulated in small pre-B cells (Figure 6B).

Mutation in the Erk-Mediated Phosphorylation Sites of Elk and CREB Inhibits Pre-BCR-Dependent Cell Expansion

Having demonstrated the potential importance of *Myc*, *Ilf2*, *Mef2c*, and *Mef2d* for pro-B cell expansion, we next wished to determine how Erk regulates transcription of these genes. In this regard, the existence of several Elk, Ets, and CREB binding

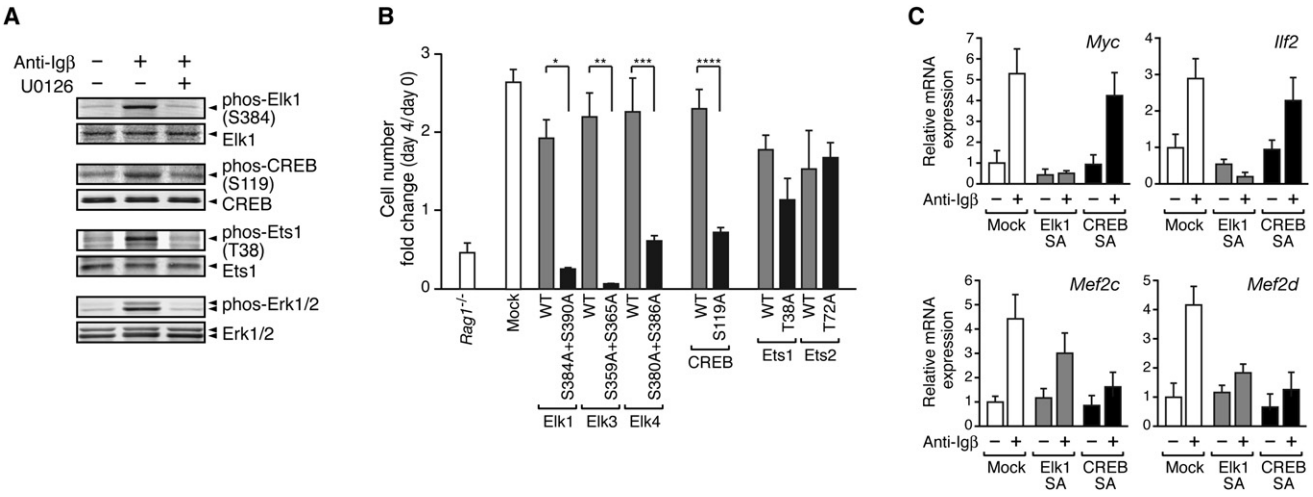

Figure 7. Phosphorylation of Elk and CREB Is Required for Pre-BCR-Mediated Cell Expansion

(A) B220$^+$ pro-B cells from *Rag1*$^{-/-}$ mice were stimulated with Igβ antibody for 3 min in the absence or presence of U0126 (20 μM). Whole-cell lysate was separated by SDS-PAGE and immunoblotted with the indicated antibodies.

(B) B220$^+$CD43$^+$ pro-B cells from wild-type mice were infected with the indicated retrovirus. The data show the fold change of cell number for each GFP$^+$ transfectant during 4 days of culture in the presence of 0.01 ng/ml of IL-7. Data are representative of three independent experiments and presented as the mean ± SD of triplicate wells. As the pre-BCR negative control, fold change of cell numbers in *Rag1*$^{-/-}$ pro-B cells infected with mock is shown (left edge). *p = 0.006, **p = 0.006, ***p = 0.01, and ****p = 0.005.

(C) B220$^+$ pro-B cells from *Rag1*$^{-/-}$ mice were infected with the indicated retrovirus. After 24 hr, GFP$^+$ cells were sorted and stimulated with Igβ antibody for 60 min. Total RNA was analyzed by quantitative PCR, with the expression of *Myc*, *Ilf2*, *Mef2c*, or *Mef2d* normalized to that of *Gapdh*. Data are representative of three independent experiments and shown as the mean ± SD of triplicate.

sites in these promoter regions allows us to hypothesize that pre-BCR activates Erk, which in turn phosphorylates Elk, Ets, and/or CREB, directly or indirectly (Figure S5). Then, phosphorylation of these factors could activate their own transcriptional activity, leading to upregulation of mRNA of *Myc*, *Ilf2*, *Mef2c*, and *Mef2d* and thereby promoting B cell expansion.

Activated Erk is known to phosphorylate the C-terminal portion of Elk-family transcription factors (two serine residues) and N-terminal portion of Ets-family transcription factors (one threonine residue); then, phosphorylated Elk and Ets are transcriptionally activated (Buchwalter et al., 2004; Yang et al., 1996). CREB belongs to the basic leucine zipper family of transcription factors, and the transcriptional activity of CREB is reported to require phosphorylation at Ser119 (Gonzalez and Montminy, 1989). Activation of Erk contributes to the phosphorylation of CREB Ser119 via the Erk-dependent serine and threonine kinase RSK (Roux and Blenis, 2004). Therefore, we examined whether Elk1, Ets1, and CREB are phosphorylated after activation of pro-B cells by anti-Igβ crosslinking. By using antibodies that specifically detected phosphorylated Elk1 at Ser384, phosphorylated CREB at Ser119, and phosphorylated Ets1 at Thr38, we found that after anti-Igβ stimulation, such a phosphorylation indeed increased in an Erk-dependent manner (Figure 7A). To determine whether phosphorylation of Elk1, Elk3, Elk4, CREB, Ets1, and Ets2 participates in pro-B cell expansion, we generated dominant-negative forms of these factors in which the Erk-mediated phosphorylation sites are mutated to Ala and infected them into pro-B cells for evaluating pro-B cell expansion. Whereas wild-type pro-B cells infected with mock retrovirus showed efficient expansion 4 days after infection, mock infected *Rag1*$^{-/-}$ pro-B cells could not expand (Figure 7B). The difference in the expansion of wild-type and *Rag1*$^{-/-}$ cells under these culture conditions was due to the lack of expression of pre-BCR, and indeed forced expression of surface pre-BCR on *Rag1*$^{-/-}$ cells resulted in an efficient expansion under the same conditions (data not shown). Forced expression of wild-type forms of Elk-1, Elk-3, Elk-4, CREB, Ets1, and Ets2 did not affect B cell expansion as much. In contrast, expression of dominant-negative mutants of Elk1, Elk3, Elk4, or CREB strongly inhibited cell expansion, suggesting the importance of the Erk-mediated phosphorylation sites of these factors in pro-B cell expansion (Figure 7B). On the other hand, transduction of pro-B cells with a dominant-negative form of Ets1 or Ets2 marginally affected the expansion.

To further test whether phosphorylation of Elk1 and CREB affects expression of *Myc*, *Ilf2*, *Mef2c*, and *Mef2d*, we introduced the above dominant-negative mutant of Elk1 or CREB into *Rag1*$^{-/-}$ pro-B cells, and the expression status of *Myc*, *Ilf2*, *Mef2c*, and *Mef2d* was determined after anti-Igβ stimulation. As shown in Figure 7C, forced expression of Elk1 S384A+S390A completely inhibited the induction of *Myc* and *Ilf2* transcripts. In contrast, forced expression of CREB S119A severely suppressed the induction of *Mef2c* and *Mef2d* transcripts. Collectively, these data suggest that Elk1 and CREB, after being phosphorylated directly or indirectly by Erk, contribute to upregulation of *Myc*, *Ilf2*, *Mef2c*, and *Mef2d*, thereby promoting pro-B cell expansion.

DISCUSSION

Here, we have addressed whether Erk signaling plays a substantial role in the pre-BCR-regulated checkpoint in B cell development and how these kinases exert their function, if any. Our

experiments demonstrate that pre-BCR induces Erk activation in an apparently ligand-independent manner, which in turn is critical for pre-BCR-mediated cell expansion. In addition, our data suggest that the activated Erk kinases mediate phosphorylation of transcription factors such as Elk1 and CREB, thereby regulating their potential target genes and promoting pre-BCR-mediated expansion (Figure S6). In contrast to direct phosphorylation of the Elk family by Erk, CREB is most probably phosphorylated by Erk-activated RSK (Roux and Blenis, 2004).

Previous reports suggest the existence of two separate pathways in pre-BCR signaling, the SFK dependent and the Syk and Zap70 dependent pathways that are crucial in pre-B cell development through NF-κB-dependent and -independent mechanisms, respectively (Schweighoffer et al., 2003; Saijo et al., 2003). Here, two lines of evidence allow us to conclude that the Erk-dependent mechanism is operating downstream of Syk and Zap70, and this in turn contributes to pre-B cell development. First, Igβ-mediated Erk activation is completely inhibited in Syk Zap70 double-deficient pro-B cells, and second, pre-BCR-mediated expansion is restored by introduction of constitutive active MEK in Syk Zap70 double-deficient cells. The latter evidence further suggests that the Erk pathway is not only necessary but also appears to be sufficient for mediating the proliferative function of the pre-BCR. The question then arises of the mechanism connecting Syk/Zap70 and Erk1/2. *Lat*$^{-/-}$ *Blnk*$^{-/-}$ and *Plcg1*$^{+/-}$ *Plcg2*$^{-/-}$ mice exhibit a similar early B cell developmental arrest (as assessed by CD43 expression) as p(I)p(C)-treated inducible-Erk double-deficient mice (Su and Jumaa, 2003; Wen et al., 2004). Moreover, in mature B cells, BCR-mediated Ras and subsequent Erk activation are severely affected, although not completely, in PLCγ2-deficient B cells (Bell et al., 2004). Thus, assuming that a similar signaling cascade takes place in the case of pre-BCR, these data suggest that SLP-76 and BLNK, after being phosphorylated by Syk and Zap70, participate in the pre-BCR-mediated PLCγ activation, thereby contributing to the Ras-Erk pathway. According to this model, RasGRPs could connect the PLCγ activity to the Ras and Erk activation because these molecules have a C1 domain that binds to DAG, a product of PLCγ (Oh-hora et al., 2003). Because a recent report suggests that Sos, another GEF for Ras, can be activated by ITAM-containing receptors, Sos could also contribute to the Ras-Erk pathway as well (Roose et al., 2007).

In contrast to loss of in vitro expansion of Erk double-deficient pro- and pre-B cells, *Btk*$^{-/-}$ or *Blnk*$^{-/-}$ pre-B cells exhibited a 10- to 15-fold increase in vitro expansion of pre-B cells (Middendorp et al., 2002; Flemming et al., 2003). This hyperexpansion is probably due to dysregulated pre-BCR signaling because this anomaly was abrogated in λ5 BLNK double-deficient B cells (Flemming et al., 2003). These data imply two things: First, given the evidence that PLCγ activation is decreased, but still occurs, in *Btk*$^{-/-}$ or *Blnk*$^{-/-}$ pre-B cells, this residual PLCγ activation might be enough to induce a certain degree of Ras and Erk activation required for pre-B cell expansion. Second, Btk and BLNK might possess antiproliferative functions for instance through downregulation of surrogate light chains and/or IL-7R components, in addition to activating PLCγ2. According to this scenario, a defect in such antiproliferative functions might be dominantly manifested in these knockout mice.

Our results demonstrate that the Elk family and CREB and their target genes constitute a link between Erk signaling and the transcriptional programs required to execute pre-B cell expansion. Indeed, phosphorylation of both Elk1 and CREB was apparently enhanced in large cycling pre-B cell stage (data not shown). By using dominant-negative interfering mutants of Elk1 and CREB in which Erk-mediated phosphorylation sites are changed to Ala, we argue that Elk1 activates expression of *Myc* and *Ilf2*, preferentially, whereas CREB regulates *Mef2c* and *Mef2d* in the pre-BCR signaling context. Because the dominant-negative approach may cause undesired side effects, these results should be carefully interpreted. However, the following three lines of the evidence seem to substantiate our idea: First, like effects of such dominant-negative forms of Elk1 and CREB, expression of *Myc*, *Ilf2*, *Mef2c*, and *Mef2d* genes was totally dependent on Erk activation. Second, *Myc* and *Ilf2* promoter regions contain multiple Elk and Ets binding sites, whereas *Mef2c* and *Mef2d* have multiple CREB binding sites. Finally, our chromatin-immunoprecipitation assays indicate that Elk1 binds to the *Myc* and *Ilf2* promoter, constitutively and inducibly, respectively, after anti-Igβ stimulation, whereas CREB binds to the *Mef2c* and *Mef2d* promoter constitutively (Figure S7). The inducible binding of Elk1 to the *Ilf2* promoter region is somewhat unexpected. Because this binding was also blocked by treatment of a MEK inhibitor (Figure S7), one of the straightforward explanations for this result is that Erk-mediated phosphorylation on Elk1 might regulate its accessibility to the *Ilf2* promoter region, in addition to promoting the transcriptional activity of Elk1.

The importance of Erk-mediated phosphorylation on CREB in a pre-BCR checking point is supported by a previous report. Transgenic mice overexpressing a dominant-negative CREB (Ser119-to-Ala mutant) in a B cell-specific manner showed a developmental block in the transition of pro- to pre-B cells (Chen et al., 2006). In contrast to CREB, single deficiency of the Elk members Elk1, Elk3, and Elk4 resulted in apparently normal B cell development, although this has not been analyzed in detail (Cesari et al., 2004; Costello et al., 2004). However, in light of the structural similarities among these members, it is still possible that a certain degree of functional compensation between them may operate in B cells, thereby tempering the phenotype. Indeed, in our assay systems, dominant-negative forms of Elk3 and Elk4 were similar to Elk1 in terms of their inhibitory potentials (data not shown).

Accumulating evidence gathered from different experimental systems indicate that c-Myc regulates the mitogenic induction of cell growth (Pelengaris et al., 2002). Here, we extend this evidence to include pre-BCR-activated B cells. In accord with our findings, a recent report has demonstrated that overexpression of c-Myc stimulated expansion of "pre-B"-like cells from pro-B cells in the absence of pre-BCR formation (in *Rag2*$^{-/-}$ mice) (Habib et al., 2007). Furthermore, this report has shown that B cell-specific deletion of c-Myc and N-Myc inhibited B cell development at the pro- to pre-B cell transition. In mature primary B cells, BCR and LPS-mediated upregulation of *Myc* transcript was severely affected in the presence of LY294002 (PI3K inhibitor) or in the absence of NF-κB1 and c-Rel, whereas treatment of PD98059 (MEK inhibitor) did not affect the upregulation (Grumont et al., 2002). Although we did not determine whether the PI3K pathway is also involved in pre-BCR-mediated *Myc* transcription, it is likely that pre-BCR utilizes the Erk pathway, in addition to the PI3K-dependent NF-κB pathway, to

induce a robust increase of *Myc* transcript. In fact, treatment of a MEK inhibitor did not completely abrogate Igβ-mediated upregulation of *Myc* in pro-B cells (Figures S4B and S4C).

Although MEF2 was initially identified as a regulator of muscle-gene expression, its importance in T cell biology has also been demonstrated. *Drosophila* possesses a single *Mef2* gene, whereas vertebrates have four—*Mef2a*, *Mef2b*, *Mef2c*, and *Mef2d*. Nur77 is a crucial mediator of TCR-induced apoptosis, and expression of *Nur77* is mediated through two MEF2 sites in the *Nur77* promoter. The following observations together suggest the involvement of MEF2 proteins in thymocyte negative selection. In unstimulated T cells, MEF2B is associated with transcriptional corepressors, such as HDAC7 and Cabin1, that inhibit *Nur77* expression (Youn and Liu, 2000). After TCR activation, HDAC7 becomes dissociated from MEF2D through nucleocytoplasmic shuttling. Phosphorylation of HDAC7 by PKD1, a PI3K-activated kinase, recruits 14-3-3 and translocates HDAC7 to the cytoplasm, allowing the activation of MEF2 proteins (Dequiedt et al., 2005). If a similar regulation occurs in the case of BCR and pre-BCR signaling, our findings implicate the existence of another layer of regulation of MEF2 family genes, namely their transcriptional regulation by the Erk pathway. In *Drosophila*, MEF2 regulates a striking number of genes that encode components of various signaling pathways including the fibroblast growth factor and epidermal growth factor (Sandmann et al., 2006). This allows us to suggest that the pre-BCR signaling components could be MEF2C and MEF2D targets, thereby enhancing pre-B cell expansion. In contrast with c-Myc, MEF2C, and MEF2D, very little is known on the role of ILF2 in lymphocyte function, except that ILF2 regulates transcription of IL-2 in cultured T cells (Zhao et al., 2005).

Our results suggest that the poor expansion of pre-B cells due to insufficient upregulation of *Myc*, *Ilf2*, *Mef2c*, and *Mef2d* might be one reason for the observed developmental defect in the absence of both Erk1 and Erk2. This proposal, however, does not preclude the following possibilities: First, gene products downregulated by pre-BCR in an Erk-dependent manner might also contribute to pre-B cell expansion. In fibroblasts, *Tob1* and *Ddit3* are candidate genes. Expression of antiproliferative genes *Tob1* and *Ddit3* are downregulated after FGF stimulation (Yamamoto et al., 2006). Second, Ras-Erk signaling pathway is thought to be important for cell survival by transcription-dependent and -independent mechanisms (Bonni et al., 1999). In fact, Erk double-deficient pro-B cells were more susceptible to cell death than wild-type cells (Figure S2A). We also found that transcription of *Bcl2* prosurvival gene was activated by anti-Igβ stimulation of μMT pro-B cells in an Erk-dependent manner (data not shown). Given the previous evidence that phosphorylation at S119 of CREB induces *Bcl2* expression during B cell activation and rescue from apoptosis (Zhang et al., 2002), it is reasonable to anticipate that this Erk-mediated survival defect in the Erk double-deficient mice could, at least partly, contribute to the defective expansion and subsequent developmental block. Third, apart from transcriptional regulation, Erk-mediated phosphorylation might directly regulate the components of the cell cycle and/or survival system, leading to pre-B cell expansion. For instance, the observed survival defect in the absence of Erk1 and Erk2 might be explained by insufficient phosphorylation of the proapoptotic product Bim, a protein known to undergo ubiquitination and subsequent proteasomal degradation upon Erk-mediated phosphorylation (Craxton et al., 2005). In this regard, in addition to the transcriptional regulation of *Myc* by Erk discussed above, the Erk pathway could regulate c-Myc protein stability by its phosphorylation (Sears et al., 2000).

The pre-BCR signal is thought to induce several rounds of proliferation and to direct epigenetic changes resulting in the allelic exclusion of the heavy-chain locus and in the activation of the light-chain loci for V(D)J recombination. Developmental blockade from pro-B to pre-B cells in the Erk double-deficient mice has enabled us to propose the connecting mechanism between Erk and pre-B cell expansion. However, whether the Erk kinases participate in allelic exclusion and light-chain gene rearrangement remains to be determined.

EXPERIMENTAL PROCEDURES

Mice

Mapk1$^{fl/fl}$ mice and *Mapk3*$^{-/-}$ mice have been described previously (Hatano et al., 2003; Pagès et al., 1999). These mice were crossed to Mx-cre mice for generation of *Mx-cre*$^{+}$ *Mapk3*$^{+/-}$ *Mapk1*$^{fl/fl}$ or *Mx-cre*$^{+}$ *Mapk3*$^{-/-}$ *Mapk1*$^{fl/fl}$ mice (Kuhn et al., 1995). To achieve the deletion of *Mapk1* flox allele, we induced Cre expression by three i.p. injections of p(l)p(C) (400 μg in PBS; Sigma) every third day. μMT and *Il7r*$^{-/-}$ mice have been described previously (Kitamura et al., 1991; Maki et al., 1996). The generation of *Syk*$^{fl/fl}$ mice is described in detail in the Supplemental Data. We crossed *Syk*$^{fl/fl}$ mice to mb1-cre mice and *Zap70*$^{-/-}$ mice to generate *mb1-cre*$^{+}$ *Syk*$^{fl/fl}$ *Zap70*$^{-/-}$ mice (Hobeika et al., 2006; Negishi et al., 1995). The mb1-cre mice were kindly provided as a gift from Dr. Michael Reth. Mice were bred and maintained under specific pathogen-free conditions and analyzed at the age of 7–16 weeks. Animal care and experiments were conducted according to the guidelines established by the RIKEN animal committee.

Radiation Chimeras

For Figures 2 and 4C, recipient mice were irradiated with a dose of 600 rad; this was followed by i.v. injection of 5×10^6 donor bone-marrow cells. Mice were given 0.16% neomycin-sulfate (Sigma) in their drinking water for 2 weeks after transfer. Two weeks after transfer, mice were given three i.p. injections of p(l)p(C) and analyzed 2 weeks after the first p(l)p(C) injection. Radiation chimeras were generated with BALB/c background *Rag1*$^{-/-}$ recipient mice (Ly9.1^{+}) and donor bone-marrow cells on the C57BL/6 (Ly9.1^{-}) background. Anti-Igβ in vivo stimulation (Figure 4C) was performed as previously described (Nagata et al., 1997).

Flow Cytometry and Cell Sorting

Single cells were stained with various fluorochrome-conjugated antibodies purchased from BD PharMingen or eBioscience (see Supplemental Experimental Procedures). Dead cells were excluded from the analysis with propidium iodide. For cell-cycle analysis, bone-marrow cells pulsed with 10 μM of bromodeoxyuridine (BrdU) for 2 hr of culture were stained with antibodies to B220, CD43, and BrdU, then labeled with 7-amino-actinocycin D (7-AAD). All analyses were made with a FACSCalibur (BD Biosciences). Specific cell populations were either sorted on a FACSVantage (BD Biosciences) or isolated by magnetic cell sorting with B220 or CD19 microbeads (Miltenyi Biotech).

Retroviral Transduction

Retroviral vector expressing membrane μH was described previously (Kawano et al., 2006). cDNA fragments were obtained from mouse splenic B cells by PCR amplification with the primers listed in Table S1. These cDNAs were verified by sequencing analysis and inserted into pMX-IRES-GFP vector as described in the Supplemental Experimental Procedures. Retroviral infection was described previously (Kawano et al., 2006).

In Vitro Pro-B Cell Culture

Cells were grown in Iscove's Modified Dulbecco's Medium (GIBCO) supplemented with 10% FCS, 50 μM 2-ME, penicillin, streptomycin, and the

indicated factors. For [^{3}H]-thymidine incorporation assay, cells were plated in triplicate at 10^5 cells/well in 96-well plates and measured on day 2 by addition of 0.5 μCi [^{3}H]-thymidine 12 hr prior to the harvest. [^{3}H] incorporation was measured with a beta counter system (PerkinElmer). Retrovirally transduced pro-B cells were counted at the indicated day as previously described (Kawano et al., 2006).

Gene-Expression Analysis

For quantitative RT-PCR analysis, randomly primed cDNA strands were generated with reverse transcriptase II (Invitrogen). With the gene-specific primers listed in Table S2, mRNA expression was quantified by iCycler (Bio-Rad Laboratories).

Immunoblotting

CD19$^+$ pro-B cells from μMT or *mb1-cre*$^+$ *Syk*$^{fl/fl}$ *Zap70*$^{-/-}$ mice and B220$^+$ pro-B cells from *Rag1*$^{-/-}$ mice were pretreated with 30 μg/ml of biotin-conjugated Igβ mAb HM79 for 20 min on ice in the presence or absence of 20 μM of U0126. After addition of 30 μg/ml of streptavidin, the cells were incubated at 37°C for the indicated time. Cell lysates were separated on 5%–20% SDS-PAGE and transferred to a polyvinylidene difluoride membrane (Millipore). The membrane was immunoblotted with specific antibodies (see Supplemental Experimental Procedures).

Statistical Analysis

Statistical significance was calculated with the two-tailed Student's t test. All p values of less than 0.05 were considered significant.

ACCESSION NUMBERS

The microarray data have been deposited in CIBEX under accession number CBX58.

SUPPLEMENTAL DATA

Additional Experimental Procedures, seven figures, and two tables are available at http://www.immunity.com/cgi/content/full/28/4/499/DC1/.

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